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The Genetic Variability of Prolactin and Signal Transducers and Activators of Transcription 5A (STAT5A) Genes in Bali Cattle

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ABSTRACT

The aim of this study was to identify the genetic variability of PRL and STAT5A genes in Bali cattle from Bali, West Nusa Tenggara (NTB), and South Sulawesi (SS) using PCR-RFLP method. A total of 262 Bali cattle were identified using *Rsa*I (PRL) and *Ava*I (STAT5A) restriction enzymes. PRL gene exon 3, PRL gene exon 4, and STAT5A gene exon 7 amplifications resulted fragments with the lengths of 156 bp, 294 bp, 215 bp, respectively. Genotyping of PRL gene both at exon 3 and 4 produced three genotypes in Bali population and two genotypes in NTB and SS population. For the PRL gene, frequencies of A allele (exon 3) and G allele (exon 4) were dominant to the B allele (exon 3) and A allele (exon 4) across all populations. The results showed that STAT5A|*Ava*I loci had monomorphic C allele. Heterozygosity values were found low at both exons 3 and 4 of PRL gene in all population. Sequence analysis results of PRL gene both for exons 3 and 4 showed that there was a mutation between adenine (A) and guanine (G) bases in the *Rsa*I recognized site, whereas in STAT5A gene we can confirm the existence of *Ava*I restriction site (C|CCGAG).

Key words: bali cattle, genetic polymorphism, PCR-RFLP, prolactin gene, STAT5A gene

ABSTRAK

Penelitian ini bertujuan untuk mengidentifikasi ada tidaknya polimorfisme gen PRL dan STAT5A pada sapi bali di tiga daerah pusat pembibitan sapi bali, yaitu: Bali, Nusa Tenggara Barat (NTB), dan Sulawesi Selatan (SS) dengan menggunakan metode PCR-RFLP. Sapi bali sebanyak 262 sampel dideteksi keragaman genetiknya menggunakan enzim restriksi *Rsa*I (PRL) dan *Ava*I (STAT5A). Amplifikasi gen PRL ekson 3, gen PRL ekson 4, dan gen STAT5A ekson 7 menghasilkan fragmen dengan panjang masing-masing 156 bp, 294 bp, dan 215 bp. Penentuan genotipe gen PRL ekson 3 dan ekson 4 menghasilkan tiga genotipe pada populasi di Bali dan dua genotipe pada populasi di NTB dan SS. Frekuensi alel A (ekson 3) dan alel G (ekson 4) lebih tinggi dibandingkan dengan alel B (ekson 3) dan alel A (ekson 4) untuk gen PRL. Hasil analisis menunjukkan bahwa lokus STAT5A|*Ava*I memiliki alel monomorfik C. Nilai heterozigositas ditemukan rendah pada gen PRL ekson 3 dan ekson 4 untuk seluruh populasi. Hasil sekuens fragmen gen PRL baik pada ekson 3 maupun ekson 4 menunjukkan adanya mutasi antara basa adenin (A) dengan guanin (G), sedangkan hasil analisis sekuens pada gen STAT5A dapat mengonfirmasi adanya situs restriksi enzim *Ava*I (C|CCGAG).

Kata kunci: gen prolaktin, gen STAT5A, keragaman genetik, PCR-RFLP, sapi bali

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INTRODUCTION

Bali cattle (*Bos javanicus*), a domesticated descendant of the wild Banteng (*Bibos banteng*) (Payne & Rollinson, 1973) is one of Indonesian indigenous animal genetic resources. Bali cattle has advantages, one of which is its remarkable reproductive ability, especially under harsh environments and low-quality fodder (Talib, 2002). Moreover, bali cattle known as the most preferred breed of cattle in the small holding system, because of their conditions, fertilities, and low calf mortalities (Purwantara *et al*., 2012).

Bali cattle represents approximately 31% of the total local cattle population in Indonesia (DGLAH, 2011). Population of Bali cattle, beside in Bali Island itself, have been widely distributed throughout the eastern islands of Indonesia. There were three major populations of bali cattle: South Sulawesi, East and West Nusa Tenggara, and Bali Island. Strategies to support implementation of conservation and breeding programmes can be made

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through the role of breeding centre in order to produce superior bali cattle breeding stock. This study focused in the three bali cattle breeding centres in Indonesia: West Nusa Tenggara (BPT-HMT Serading), South Sulawesi (Barru District), and Bali Island (Breeding Centre of bali cattle, Singosari Artificial Insemination Centre, and Baturiti District Artificial Insemination Centre).

Improvement of bali cattle genetic quality can be done by selection to obtain desirable genetic characteristics of Bali cattle, i.e. its high reproductive ability. Previous study reported that Bali cattle had high fertility rate, about 69%-83% (Talib, 2002), services per conception was 1.65±0.87 (Siswanto *et al*., 2013), ages at first calving were 43.86±0.70 mo (Gunawan *et al*., 2011) and 1104.51±23.82 d (Siswanto *et al*., 2013), and calving rates were 350.46±27.98 d (Siswanto *et al*., 2013) and 360.93±4.47 d (Gunawan *et al*., 2011).

Khatib *et al*. (2009) stated that there were several candidate genes that affected reproductive traits, some of which were prolactin gene and STAT5A gene. Prolactin (PRL) and signal transducers and activators of transcription 5A (STAT5A) are members of POU1F1 signaling pathway related to reproductive traits. Single gene analysis revealed significant associations of STAT5A gene with embryonic survival and fertilization rate. Moreover, the 2-ways interactions of POU1F1 and PRL genes analysis were found to be associated with significant effects on early embryonic survival

Prolactin is a polypeptide hormone, mainly synthesized and secreted by the lactotroph cells of the pituitary gland, but also produced by extrapituitary sites such as mammary gland, placenta, uterus, and T lymphocytes (Bachelot & Binart, 2007). The bovine PRL gene was a candidate gene which is associated with reproduction and milk performance traits (Brym *et al*., 2005) because its role in female fertility, mammogenesis, lactogenesis, and galactopoiesis. The bPRL gene resides at chromosome 23 (Barendse *et al*., 1997), consists of five exons and separated by four introns. Single nucleotide polymorphism (SNP) of Adenine (A) - G (Guanine) was reported to give rise to a polymorphic PRL|*Rsa*I site (Brym *et al*. 2005). Many studies in cattle breeds from various countries have demonstrated the association of PRL polymorphism with milk yield, fat percentage, and milk protein content (Brym *et al.*, 2005; Dong *et al*., 2013; Mehmannavaz *et al*., 2009; Alipanah *et al*., 2007; Dybus *et al*., 2005; Lazebnaya *et al*., 2013). PRL|*Rsa*I loci has been widely used as a marker for genetic characterization of cattle population.

Signal transducers and activators of transcription (STAT) known as mammary gland factor (MGF), was discovered as intracellular mediator of prolactin signaling and can activate transcription of milk protein genes in response to prolactin (Wakao *et al*., 1994). The bovine STAT5A gene localized in chromosome 19, consists of 19 exons coding for 794 amino acid chains (Seyfert *et al*., 2000). Flisikowski *et al.* (2003) reported that SNP within exon 7 of the bovine STAT5A gene as a substitution between cytosine and thymine at position of 6853 (C6853T). This mutation creates *Ava*I restriction site which can be detected by using PCR-RFLP method. Genetic polymorphisms of STAT5A gene at exon 7 were associated with meat production traits (Flisikowski *et al*., 2003), growth performance traits (Dario *et al.,* 2009b), and milk production traits (Sadeghi *et al*., 2009).

Genetic characterization of reproductive traits marker was suggested to complete the base information in order to improve implementation of breeding and conservation of bali cattle. Identifications of genetic diversity in bali cattle were reported in previous study: GH|*Alu*I loci by Jakaria & Noor (2011), GHR|*Alu*I loci by Zulkharnaim *et al*. (2010), SNP in exon 4 of IGF-1 by Maskur *et al.* (2012). Until now, the characterization of PRL and STAT5A genes in Bali cattle has never been conducted, therefore the information of these candidate genes was not available. The objective of this research was to identify the genetic variabilty of PRL and STAT5A genes polymorphisms in bali cattle at three breeding centre regions (Bali, West Nusa Tenggara, and South Sulawesi) by using the PCR-RFLP method followed by direct sequencing.

MATERIALS AND METHODS

Sample Sources

The total numbers of bali cattle used in this study were 262 samples derived from three populations. Samples from Bali Island were taken from Breeding Centre of bali cattle (100 heads), Baturiti District Artificial Insemination Centre (22 heads), and Singosari Artificial Insemination Centre (28 heads). Samples from West Nusa Tenggara (NTB) were taken from BPT-HMT Serading (48 heads). Samples from South Sulawesi were taken from Barru District (64 heads). Samples were analyzed in the form of blood samples, semen samples, and DNA collections from Livestock Molecular Genetics Laboratory, Faculty of Animal Science, Bogor Agricultural University.

DNA Extraction

Genomic DNA was extracted from blood samples and semen samples by using phenol-chloroform method followed by ethanol precipitation (Sambrook *et al*. 1989) and the DNA was dissolved in elution buffer. The quality of the total genome extraction was performed by 1% agarose gel electrophoresis.

DNA Amplification

Three pairs of PCR primer were designed to amplify parts of exon 3 and exon 4 of PRL gene, and exon 7 of STAT5A gene, as described by Mitra *et al*. (1995), Brym *et al*. (2005), and Flisikowski *et al*. (2003), respectively (Table 1). The PCR was carried out in a reaction volume of 15 µL containing 1 µL genomic DNA template, 0.2 µL each primer, 0.3 µL dNTPs (Thermo Scientific), 1 µL $MgCl_2$, 0.05 µL Taq polymerase (Thermo Scientific), 1.5 µL 10xbuffer (Thermo Scientific), and 10.75 µL distillated water. Amplification was carried out with a thermal cycler machine GeneAmp® PCR System 9700 (Applied Biosystem). The condition of thermal cycling consisted of predenaturation at 95 ^{o}C for 5 min, followed

Table 1. Forward and reverse primers sequences for the amplification

Gene	Sequence	Product size	References
STAT5A exon 7	F: 5′-CTG CAG TGG CGT TCT GAG AG-3′ R: 5′-TGG TAC CAG GAC TGT AGC ACA T-3′	215 bp	Flisikowski *et al*. (2003)
PRL exon 3	F: 5′-CGA GTC CTT ATG AGC TTG ATT CTT-3′ R: 5′-GCC TTC CAG AAG TCG TTT GTT TTC-3′	156 bp	Mitra *et al*. (1995)
PRL exon 4	F: 5′-CCA AAT CCA CTG AAT TAT GCT T-3′ R: 5′-ACA GAA ATC ACC TCT CTC ATT CA-3′	294 bp	Brym *et al*. (2005)

by 35 cycles of denaturation at 95 ^oC for 10 s, annealing at 60 ^oC for 20 s, and extension at 72 ^oC for 30 s. The final extension step was at 72 ^oC for 5 min. The DNA amplification products were checked on 1.5% agarose gels in 0.5 x TBE buffer containing with a 100 bp ladder as a molecular weight marker.

Genotyping by PCR-RFLP

For the PCR-RFLP analysis, 5 µl each PCR product was digested with 0.3 µL *Rsa*I enzyme for PRL gene and *Ava*I enzyme for STAT5A gene, 0.7 µL Tango buffer, and 1 µl distillated water at 37 ^oC for 16 h. A 100 bp molecular weight marker (for PRL gene at exon 4 and STAT5A gene) and a 20 bp molecular weight marker (for PRL gene at exon 3) were used to determine the length of digestion product. The digestion products were separated on 2% agarose gels (for PRL gene at exon 4 and STAT5A gene) and 3.5% agarose gels (for PRL gene at exon 3) in 0.5 x TBE buffer by electrophoresing at 100 volt for 45 min and visualized on UV transiluminator.

DNA Sequencing and Analysis

PCR products representing different genotype of each gene were sequenced with DNA sequencer machine provided by DNA sequencing service in 1st Base. Sequence results were analyzed by BioEdit (Hall, 1999) and sequence alignment was performed by using MEGA software version 5.0 (Tamura *et al*., 2011) in order to find nucleotide mutation. The BLAST (Basic Local Alignment Search Tool) program was used to search the NCBI GenBank databases for reference and homologous sequences.

Data Analysis

Genotype and allele frequencies. The genotype and allele frequencies as were described by Nei & Kumar (2000) were analyzed by using genotyping data from each gene and calculated based on the populations of Bali cattle (Bali, NTB, and South Sulawesi). Genotype frequency was calculated by the following formula:

$X_{ii}= n_{ii}/N$

Allele frequency was calculated by the following formula:

$X_i= (2n_{ii} + \sum n_{ij})/2N$

Description:
X_{ii} = iith genotype frequency
X_i = ith allele frequency
n_{ii} = Number of sample of ij genotype
n_ij = Number of sample of ij genotype
N = Total samples

Heterozygosity. Observed heterozygosity (Weir, 1996) and expected heterozygosity (Nei & Kumar, 2000) were tested by the following formula:

$$H_o=\sum_{i\neq j}\frac{n_{ij}}{N}$$

$$H_e=1-\sum_{i=1}^{q}x_i^2$$

Description:
H_o = Observed heterozygosity
n_{ij} = Number of heterozygous animal
N = Number of observed animal
H_e = Expected heterozygosity
$\mathbf{x}_i$ = Frequency of allele
q = Total alleles

Hardy-Weinberg equilibrium. Test of Hardy-Weinberg equilibrium (HWE) was conducted with chi-square test (Kaps & Lamberson, 2004) and was computed by using PopGene32 software version 1.32 (Yeh *et al*., 1999):

$$\chi^2=\sum\frac{(\text{obs-exp})^2}{\text{exp}}$$

Description:
χ^2 = Hardy Weinberg equilibrium test
obs = Observed number of iith genotype
exp = Expected number of iith genotype

The number of degrees of freedom (df) is equal to the number of possible genotypes minus the number of alleles (Allendorf *et al*., 2013) or as decribed below:

df= (number of iith genotype) – (number of jth allele)

Genetic differentiation (F_{ST}). F_{ST} was based on Nei's F-statistics (1977) and was computed by using PopGene32 software version 1.32 (Yeh *et al*., 1999):

Fst = (Ht – Hs)/Ht

Description:
Ht = expected HW heterozygosity if the entire base population were panmitic

Ho = observed proportion of heterozygosity averaged over all subpopulations
Hs = expected heterozygosity averaged over all subpopulations.

RESULTS AND DISCUSSION

Genetic Polymorphism of the PRL Gene

PRL gene and STAT5A gene fragments were succesfully amplified by using polymerase chain reaction technique for all samples. The 156 bp fragment of third exon of PRL gene, 294 bp fragment of fourth exon of PRL gene, and 215 bp fragment of seventh exon of STAT5A gene in bali cattle samples were shown in Figure 1.

PRL|*Rsa*I restriction analysis of the exon 3 region revealed three genotypic patterns in bali cattle and showed two alleles (A and B alleles) in all bali cattle population. The restriction patterns were single fragment or uncut of 156 bp (referred to AA genotype), two fragments of 82 bp and 74 bp (BB genotype), and three fragments of 156, 82, and 74 bp were the heterozygous AB genotype (Figure 2).

The genotype and allele frequencies of the third exon of PRL gene for bali cattle in all population were presented in Table 2. The homozygous AA genotype was present in the highest frequency across all the population. The frequency of AA genotype of Bali cattles in Bali (0.9133) was higher than those in NTB (0.7083) and South Sulawesi (0.8906). Frequency of the AB genotype in NTB population (0.2917) was higher than those in South Sulawesi (0.1094) and Bali (0.0801). The results showed that those bali cattle with the BB genotype were very low in Bali population (0.0066) and none of these animals had the BB genotype in NTB and South Sulawesi populations.

The distribution of the PRL|*Rsa*I alleles of the third exon is characterized by a higher frequency of the A allele compared to the B allele in most bali cattle population studied (Table 2). The frequencies of the A allele in Bali and South Sulawesi populations almost similar (0.9533 vs 0.9543) while the A allele for bali cattle

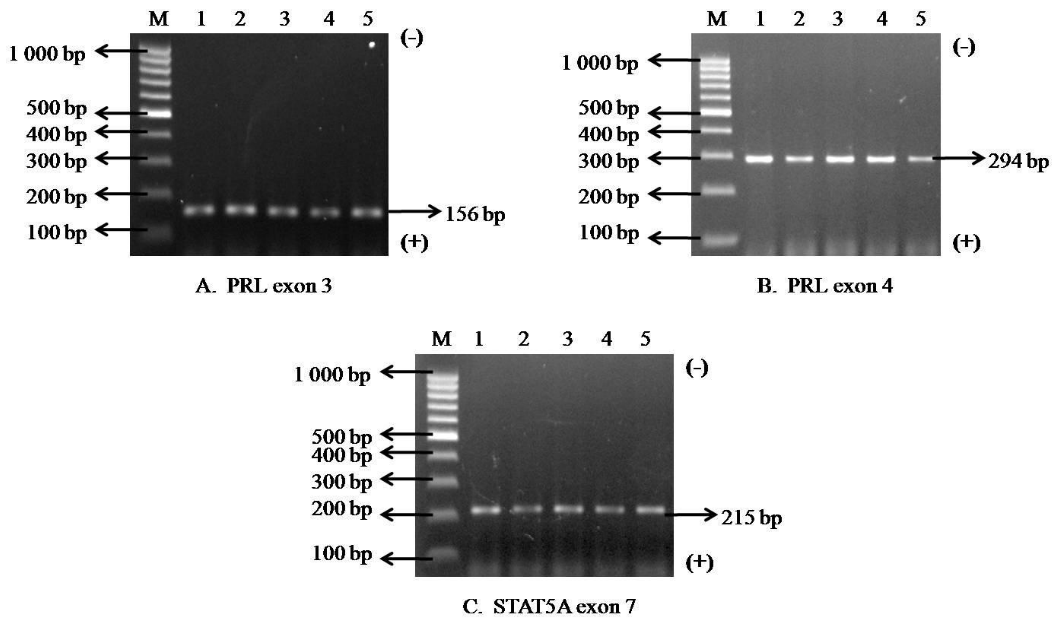

Figure 1. Visualization of PCR product amplified from bali cattle samples. (A) 1-5= PCR product of prolactin gene at exon 3 (156 bp); M= 100 bp ladder size standard. (B) 1-5= PCR product of prolactin gene at exon 4 (294 bp); M= 100 bp ladder. (C) 1-5= PCR product of STAT5A gene at exon 7 (215 bp); M= 100 bp ladder.

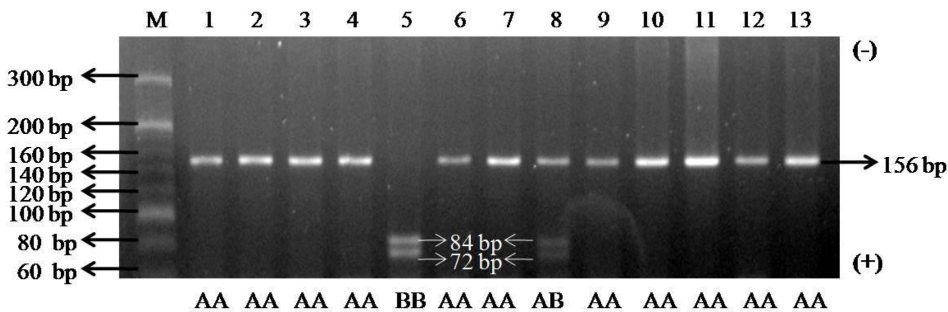

Figure 2. Restriction pattern of PRL gene at exon 3 fragment using PCR-RFLP method with *Rsa*I enzyme on 3.5% agarose gel. M= 20 bp ladder size standard; 1-13= Number of samples; AA, AB, BB= Genotype.

population in NTB was lower (0.8542) with regarding to the higher AB genotype at NTB. In general, the A allele frequency of the PRL gene at exon 3 of bali cattle identified in this study was higher than those reported in some cattle breeds such as *Bos indicus* (0.52, Sodhi *et al*., 2011), Russian Red Pied (0.794, Alipanah *et al*., 2007), Black-and-White (0.8533, Dybus *et al*., 2005), Nadji (0.571, Roshanfekr *et al*., 2013), Turkish Grey (0.762, Akyuz *et al*., 2012), Simmental (0.810, Akyuz *et al*. 2013), Holstein-Friesian (0.582, Wojdak-Maksymiec *et al*., 2008), and Aboriginal Russian (0.732, Lazebnaya *et al*., 2013). This study confirmed the predominance of the A allele across three bali cattle populations in Indonesia.

Lazebnaya *et al*. (2013) reported the association of AA and AB genotypes at PRL at exon 3|*Rsa*I locus with higher fat content of milk than BB genotype. Contrastly, Alipanah *et al*. (2007) reported that animals with BB genotype had higher milk production, milk fat yield, and milk protein yield in Russian Red Pied cattle.

RFLP (restriction fragment length polymorphism) analysis using *Rsa*I restriction enzyme was used to genotyping PRL gene of the fourth exon. Digestion of the 294 bp PCR product resulted in three restriction patterns. The first pattern, with two restriction fragments of 162 and 132 bp was referred to as AA genotype. The second pattern was lacking restriction site and showed one uncut fragment of 294 bp (GG genotype). The third pattern with all three fragments (294, 162, 132 bp) for AG heterozygotes (Figure 3).

The results of the genotype and allele frequency in bali cattle based on population were shown in Table 3. The GG genotype frequencies was higher compared with AA and AG genotypes. Frequencies of animals with GG genotype in Bali (0.9133) were the highest compared than those in NTB (0.750) and South Sulawesi (0.8750). The AG genotype frequencies in Bali (0.0801) and South Sulawesi (0.150) were lesser compared to AG genotype in NTB (0.250). Frequencies of AA genotype were very low (0.0066) in Bali, on the other hand none of these bali cattle was identified having the AA genotype at NTB and South Sulawesi. This situation could be becoming an indication for a limited number of AA genotype in bali cattle breeding centres.

The frequencies of the G allele in Bali and South Sulawesi populations almost similar (0.9533 vs 0.9375) while the G allele for bali cattle population in NTB was lower (0.8570). The low G allele (0.8750) at NTB might be due to limited number of the heterozygous AG genotype in this population. The G allele frequency of the PRL gene at exon 4 of bali cattle identified in this study was higher than those in some cattle breeds such as Chinese Holstein (0.8754, Dong *et al*., 2013; 0.893, Lü *et al*., 2010), Iranian Holstein (0.931, Mehmannavaz *et al*., 2009), Colombia Holstein (0.833, Rincon *et al*., 2011), Sahiwal (0.810, Ishaq *et al*., 2013), Achai (0.560, Ishaq *et al*., 2013), and Black-and-White, (0.887, Brym *et al*., 2005).

Brym *et al*. (2005) in their study about association of bovine prolactin gene exon 4 polymorphism with milk performance traits in Black-and-White cattle revealed that cows with AG genotype showed the highest milk yield, while cows with GG genotype showed the highest fat content. Contrastly, Dong *et al*. (2009) reported that the G allele was unfavourable for milk and protein yield.

Genetic Polymorphism of the STAT5A Gene

One genotypic pattern was produced as the result of *Ava*I enzyme digestion. The enzyme cuts the amplified 215 bp product into 181 and 34 bp fragments for allele C (Figure 4). Table 4 showed there was no genotype variation in bali cattle across all the population studied. Genotype frequencies of STAT5A gene at exon 7 were 1.00 for CC. Thus, the allele frequencies were 1.00 for C allele. The STAT5A|*Ava*I loci was in fixation because all population had equal allele frequencies or known as monomorphic allele.

Table 2. Allelic and genotypic frequencies values of PRL gene exon 3 based on population

Population	Number of samples	Genotype frequency			Allele frequency	
		AA	AB	BB	A	B
Bali	150	0.9133 (137)	0.0801 (12)	0.0066 (1)	0.9533	0.0467
NTB	48	0.7083 (34)	0.2917 (14)	0.000 (0)	0.8542	0.1458
South Sulawesi	64	0.8906 (57)	0.1094 (7)	0.000 (0)	0.9453	0.0547

Table 3. Allelic and genotypic frequencies values of PRL gene exon 4 based on population

Population	Number of samples	Genotype frequency			Allele frequency	
		AA	AG	GG	A	G
Bali	150	0.0066 (1)	0.0801 (12)	0.9133 (137)	0.0467	0.9533
NTB	48	0.000 (0)	0.2500 (12)	0.7500 (36)	0.1250	0.8570
South Sulawesi	64	0.000 (0)	0.1250 (8)	0.8750 (56)	0.0625	0.9375

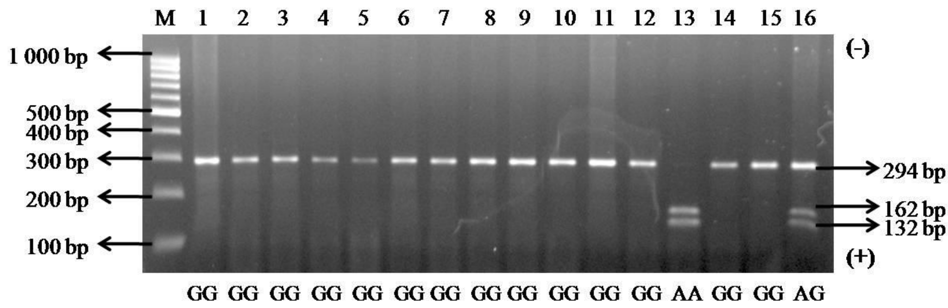

Figure 3. Restriction pattern of PRL gene at exon 4 fragment using PCR-RFLP method with *Rsa*I enzyme on 2% agarose gel. M= 100 bp ladder size standard; 1-16= Number of samples; AA, AG, GG= Genotype.

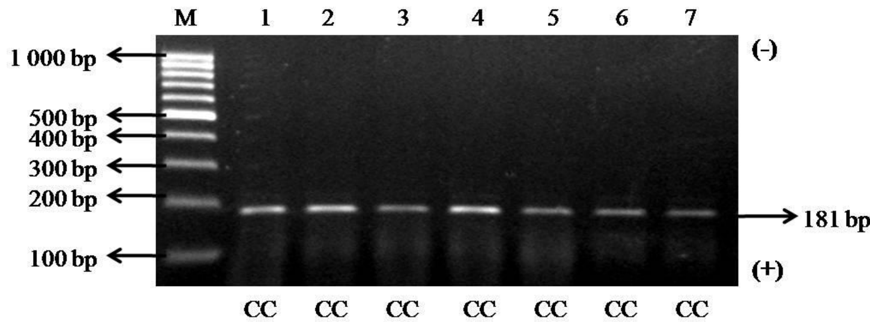

Figure 4. Restriction pattern of STAT5A gene at exon 7 fragment using PCR-RFLP method with AvaI enzyme on 2% agarose gel. M= 100 bp ladder size standard; 1-7= Number of samples; CC= Genotype.

Table 4. Allelic and genotypic frequencies values of STAT5A gene exon 7 based on population

Population	Number of samples	Genotype frequency			Allele frequency	
		CC	CT	TT	C	T
Bali	150	1.000 (150)	0.000 (0)	0.000 (0)	1.000	0.000
NTB	48	1.000 (48)	0.000 (0)	0.000 (0)	1.000	0.000
South Sulawesi	64	1.000 (64)	0.000 (0)	0.000 (0)	1.000	0.000

Genetic polymorphism of STAT5A|*Ava*I was reported in Italian cattle (Dario *et al*., 2009a), Podolica (Dario *et al*., 2009b), Jersey (Dario & Selvaggi, 2011), Polish Red-and-White (Kmiec *et al*., 2010), beef cattle breeds (Flisikowski *et al*., 2003), Italian Brown (Selvaggi *et al*., 2009), and Iranian Holstein (Sadeghi *et al*., 2009). Contrary to that, this study found STAT5A|*Ava*I locus monomorphic and fixed with C allele in all populations. This monomorphisms confirmed the predominance of the C allele in bali cattle and resulted in restrictiveness of genetic diversity usage of the STAT5A gene exon 7. Monomorphism in bali cattle were reported previously by Zulkharnaim *et al*. (2010) in GHR|*Alu*I loci, Ishak *et al*. (2011) in FSH beta-subunit|*Pst*I loci, Mu'in & Supriyantono (2012) in κ-casein|*Hind*III loci, and Jakaria & Noor (2011) in GH|*Alu*I loci.

According to Dario *et al*. (2009b), the STAT5A gene is a candidate marker for quantitative traits in livestock animals and the study reveals that CC and CT bulls show a higher live weight and a faster growth. Khatib *et al*. (2008) states that STAT5A gene is a candidate gene because it is a member of interferon-τ (IFN-τ) and placental lactogen (PL) signal transduction pathways, which plays important roles in reproduction and milk production traits.

Hardy-Weinberg Equilibrium

The result of chi-square (χ^2) test showed that the distribution of genotypes derived from third and fourth exon of PRL gene in Bali Island population was in Hardy-Weinberg equilibrium. Hardy-Weinberg equilibriums for both at exon 3 and exon 4 of PRL gene in NTB and South Sulawesi population were also in Hardy-Weinberg equilibrium (Table 5). Hardy-Weinberg equilibrium for STAT5A gene in all population could not be analyzed because the allele frequency (C) was 1.00. A certain population was in Hardy-Weinberg equilibrium if the genotype frequencies and allele frequencies were constant from one generation to the next generation

Table 5. Heterozygosity and Hardy-Weinberg equilibrium (HWE) values of bali cattle based on population

Gene	Population	Number of samples	Heterozygosity		HWE
			$H_{observed}$	$H_{expected}$	
PRL exon 3	Bali	150	0.0800	0.0893	1.738164^{ns}
	NTB	48	0.2917	0.2518	1.287865^{ns}
	South Sulawesi	64	0.1094	0.1042	0.182231^{ns}
PRL exon 4	Bali	150	0.0800	0.0893	1.738164^{ns}
	NTB	48	0.2500	0.2211	0.889845^{ns}
	South Sulawesi	64	0.1250	0.1181	0.247059^{ns}
STAT5A exon 7	Bali	150	0.0000	0.0000	undefined
	NTB	48	0.0000	0.0000	undefined
	South Sulawesi	64	0.0000	0.0000	undefined

Note: ns= not significant at level X^2 df= 1, α 5%= 3.84.

that resulted from gametes fussion randomly in large population size (Allendorf *et al*., 2013). Compared with heterozigosity values for PRL gene at exon 3 and exon 4, the genotype frequencies were also in equilbrium for all populations. The genotype frequencies equilibrium of PRL gene might suggested that selection in PRL|*Rsa*I locus was not occured in all bali cattle population studied.

Heterozygosity

According to Marson *et al*. (2005), the genetic diversity of population can be measured by using heterozygosity value with a purpose to help selection program. Heterozygosity values (Table 5) showed that PRL|*Rsa*I at exon 3 and exon 4 regions in West Nusa Tenggara (NTB) population had the highest heterozygosity values (0.2917 and 0.2500, respectively) followed by lower heterozygosity values in South Sulawesi population (0.1094 and 0.1250, respectively) while heterozygosity value in Bali population for the same locus had the lowest heterozygosity value (0.0800). Analysis results of the observed heterozygosity (H_o) and expected heterozygosity (H_e), both for exon 3 and exon 4 of PRL gene indicated that the genotype frequencies from all populations studied were in equilibrium as compared with Hardy-Weinberg equilibrium.

However, from overall heterozygosity values showed that the lowest heterozygosity value was found in STAT5A|*Ava*I locus (0.000) in all populations due to monomorphic C allele. The results of heterozygosity values in this study were low as described by Javanmard *et al*. (2005) that if the heterozygosity value was below 0.5 then the variabilty of certain gene in the population was low. Low heterozigosity values for all loci in this study indicated the occurance of inbreeding within bali cattle population studied. Different number of samples might cause the difference in heterozygosity value.

The implementation of breeding program for Bali cattle over the past years was not well directed and sustained. The largest and the best bali cattles usually slaughtered so that negative selection occured in bali cattle population. The decreasing of bali cattle genetic diversity causes alteration in the genotype proportion and only animals that have certain genotype that were available to be inherited to the next descendants. Government implemented a national program that aims to improve the genetic quality and purity of bali cattle in Bali Island since 1976 through P3 Bali (Bali Cattle Development and Breeding Project) based on Decree of The Minister of Agriculture no. 776/Kpts/Um/12/1976. This project works through selection within bali cattle breed to obtain good quality breed of bali cattle. Centralization and genetic purification of bali cattle in certain areas for long period of time led to inbreeding. Noor (2008) states that inbreeding is a form of genetic isolation due to the isolated population will lead to limitations in the choice of mating. Thus the homozygous alleles will passed on to the offspring.

Genetic Differentiation (F_{ST})

F_{ST} is the correlation between two gametes drawn at random from each subpopulation and measures the degree of genetic differentiation of subpopulations (Nei, 1977). The average genetic differentiation (Fst) among populations was 2.14% (Table 6), only PRL loci both at exon 3 and exon 4 contribute to this differentiation. With an exception, the STAT5A|*Ava*I loci is in fixation because all populations have equal allele frequencies (C allele= 1.00) or known as monomorphic allele.

Sequence Analysis of PRL Gene

Sequence analysis using forward and reverse primer revealed that PCR product of PRL gene exon 3 in this study was similar with the 156 bp in length based on previously reported by Sodhi *et al.* (2011). The results of sequence alignment compared with *Bos taurus* GenBank (accession number AY339391 and AF426315) showed that a mutation occured in exon 3 of PRL gene (Figure 5) that caused a lack of *Rsa*I restriction site (GT|AC). Lewin

Table 6. F_{ST} values of bali cattle based on loci

Gene	Number of samples	F_{ST}
PRL exon 3	262	0.0268
PRL exon 4	262	0.0159
STAT5A exon 7	262	0.0000
Means	262	0.0214

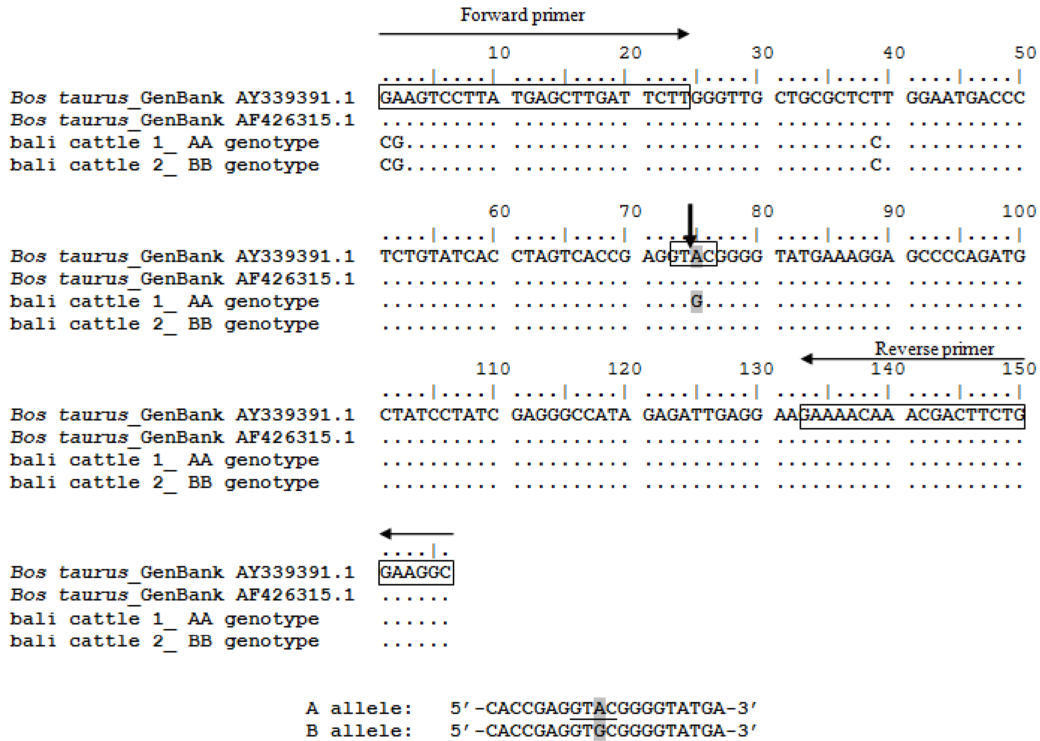

Figure 5. Nucleotide sequences alignment results of PRL gene at exon 3. GenBank of PRL sequences (accession number AY339391 and AF426315) were used for reference to find nucleotide mutation. ↓= Restriction sites of RsaI restriction enzyme (GT|AC); . . . . = Homologous sequences.

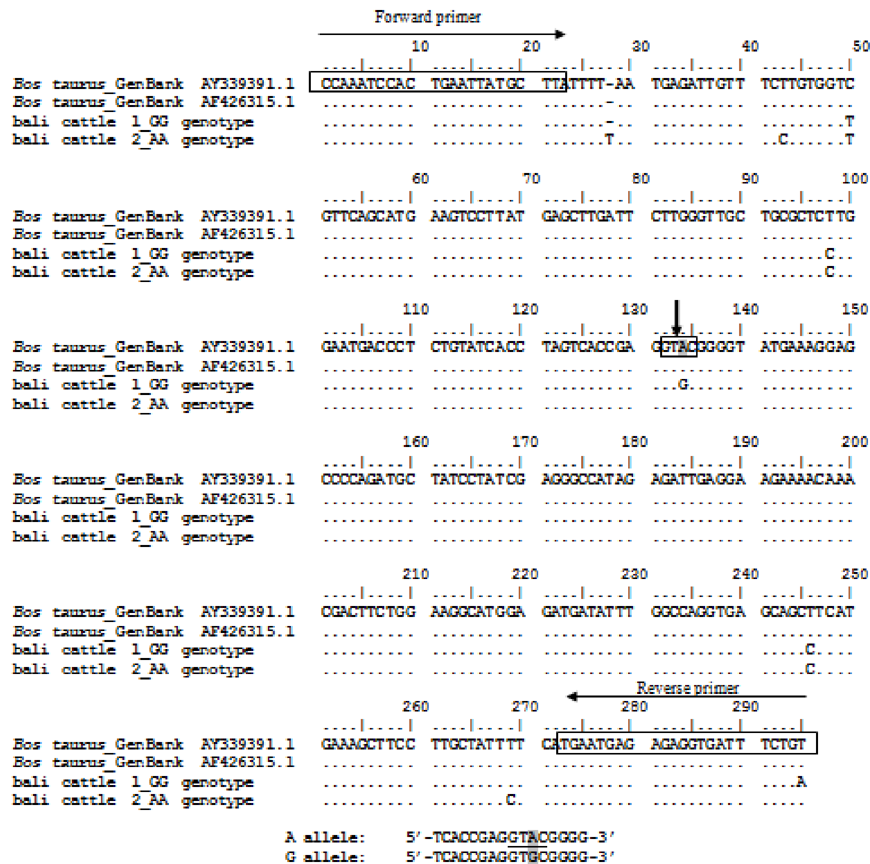

Figure 6. Nucleotide sequences alignment results of PRL gene at exon 4. GenBank of PRL sequences (accession number AY339391 and AF426315) were used for reference to find nucleotide mutation. ↓ = Restriction sites of RsaI restriction enzyme (GT|AC); . . . . = Homologous sequences.

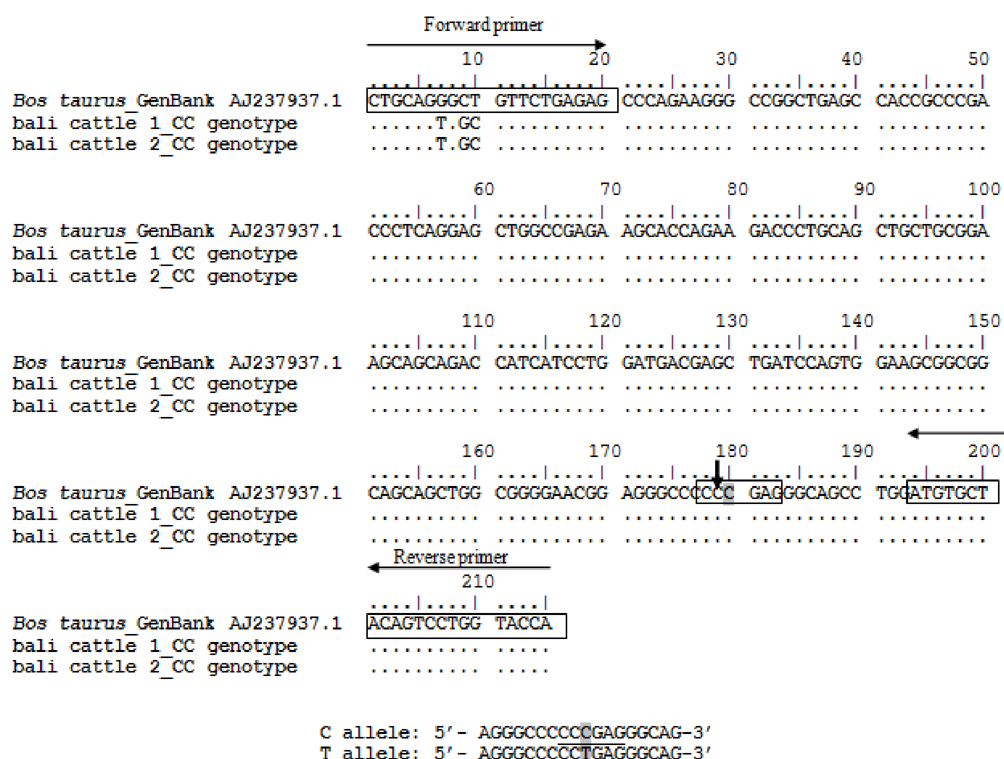

Figure 7. Nucleotide sequences alignment results of STAT5A gene at exon 7. GenBank of STAT5A sequences (accession number AJ237937) were used for reference to find nucleotide mutation. ↓ = Restriction sites of AvaI restriction enzyme (C|YCGRG); . . . . = Homologous sequences.

et al. (1992) reported that there was a synonimous A-G transition mutation found in the codon of amino acid residue 103 in the third exon of bovine PRL and resulted in a *Rsa*I polymorphic site. Transition mutation between adenine (A) base with guanine (G) base in bali cattle individual with AA genotype causing alteration in *Rsa*I restriction site sequences GT|AC became GT|GC so that the *Rsa*I enzyme couldn't recognize its restriction site.

Similar A-G transition mutation was detected in *Rsa*I restriction site in the fourth exon of PRL gene (Figure 6). The GG genotype represented mutation of A into G that produced uncut fragment in 294 bp. This result was similar to that previously described by Brym *et al*. (2005) that a transition A-G SNP (single nucleotide polymorphism) in position 8398 R including part of exon 4 of PRL gene was found by using PCR-SSCP and direct squencing methods. Association between this SNP with milk performance traits in Black-and-White cows resulted that cows with GG genotype showed higher fat contents in the first lactation and AG genotype resulted in significantly higher milk yield. Mehmannavaz *et* al. (2009) reported that the A-G mutation in PRL|*Rsa*I recognition site for both of bases is a silent mutation (Val/Val).

Sequence Analysis of STAT5A Gene

Based on sequence analysis of STAT5A gene by sequence comparison with *Bos taurus* STAT5A exon 7 fragment sequence deposited in the GenBank (accession number AJ237937.1), it was identified that there was no mutation occured in the *Ava*I restriction site (C|YCGRG). Figure 7 showed that the *Ava*I enzyme recognized its restriction sequence (C|CCGAG) and produced two fragments which were being cut, namely CC genotype or C allele. IUPAC single-letter codes were used to facilitate the definition of nucleotide sequences, which are Y symbol for pyrimindine (C or T/U) and R symbol for purine (A or G). This sequence analysis results confirmed that STAT5A|*Ava*I locus in all bali cattle population studied was monomorphic. SNP in exon 7 of the bovine STAT5A gene was a substitution of C (cytosine) and T (thymine) at position 6853. This mutation resulted in silent mutation because CCC and CCT triplets both coded for the amino acid proline (Flisikowski *et al*., 2003).

CONCLUSION

PRL|*RsaI* loci both at third and fourth exons in all populations are polymorphic, whereas STAT5A|*Ava*I loci is monomorphic and fixed with C allele. Bali cattle across all the populations for PRL gene are in low level of heterozygosity. Sequence analysis results confirm a mutation of A→G base in PRL gene and the occurance of C allele in STAT5A gene.

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